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Design and synthesis of fluorescently labeled steroidal antiestrogens

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ABSTRACT

A set of derivatives of 11β-(4-oxyphenyl)estradiol were prepared as potential fluorescent imaging agents for the evaluation of the estrogen receptor. The compounds were designed based on the established affinity and selectivity of 11β-[4-(dimethylethoxy)phenyl]estradiol (RU39411) as an estrogen receptor (ER) antagonist. The 5-(dimethylamino) naphathalene-1-sulfonyl (dansyl) and 7-nitrobenzo[c][1,2,5] oxadiaol-4-yl (NBD) moieties were selected based on their fluorescent and physicochemical properties. A convergent synthesis was developed that culminated in the [3 + 2] copper (I) assisted alkyne-azide cycloaddition coupling of the steroidal and fluorescent components. Good yields were obtained for the intermediates and final products, and the structural variations in the steroid component will permit evaluation of ER affinity and selectivity.

1. Introduction

The rationale for developing fluorescent imaging agents for the estrogen receptor lies in its close association with hormone responsive diseases, particularly breast cancer [1,2]. According to the American Cancer Society, over 266,000 cases of breast cancer were predicted for 2018, and over 40,000 women dying from the disease [3]. It is the most common cancer among women, accounting for 29% of all diagnosed cancers. Because approximately two thirds of breast cancers are dependent on estrogen and/or progesterone, agents that target the estrogen receptor (ER) play a major role in breast cancer therapy. Such strategies include the use of selective estrogen receptor modulators (SERMs), such as tamoxifen, and selective estrogen receptor downregulators (SERDs), such as fulvestrant [4–6]. At the cellular and molecular level, high spatial resolution is possible using fluorescence imaging methods, if high estrogen receptor (ER) affinity and selectivity were incorporated within the ligand. The last major review on this topic, with material through 1995 [1], provided the theoretical and practical bases for this area of research. At that time, an imaging agent that met all of the criteria had not been prepared and evaluated. We have recently reviewed the development of fluorescently labeled steroidal estrogens prepared since 1995, particularly the synthetic approaches, their evaluation as potential molecular imaging agents, and a brief discussion of their advantages/disadvantages [7]. In this study we describe our current approach to develop the "next-generation' fluorescent steroidal ER imaging agent in which we use a steroidal antiestrogen as the scaffold.

The key criteria in the design of our fluorescently labeled steroidal antiestrogens involved the incorporation of the label at a site that did not significantly reduce ER binding affinity, the use of a label that was sterically small and neutral, and a synthetic strategy that would permit easy assembly. Previous studies by Ojasso and Anstead [8,9] indicated that the 11β-position of the estradiol scaffold as the most promising site for introduction of the fluorescent label [Fig. 1].

Although not optimal as fluorophores, the dansyl- and NBD-dyes [10–14] are small, neutral entities that can be readily introduced for proof-of-principle studies [Fig. 2]. Functionalization of the fluorophore through the reactive chloro-group of NBD-Cl or through the sulfonyl chloride of dansyl chloride provides easy ligation to appropriately azido-substituted biomolecules [15,16].

Our previous studies directed toward the 11β-substituted bifunctional antiestrogens has employed a convergent approach. One arm of the synthesis focused on the preparation of the azido-substituted steroid, while the other arm generated the alkynylated imaging or therapeutic group [17–20]. In the study with the spin-labeled product [17], the introduction of the spin label resulted in only a modest reduction (approximately one order of magnitude- 40% to 4.5% and 34% t0 1.9% respectively) of binding affinity at ERα and ERβ relative to the azido precursor. Therefore, a convergent synthetic strategy by which the fluorophores could be incorporated should not only be successful but should also retain most of the receptor affinity [Fig. 3].

In this current study we chose to incorporate the 11β-(4-azidoethoxyphenyl) estradiol scaffold which we previously described as an effective ER-targeting group. To ultimately evaluate the effect of

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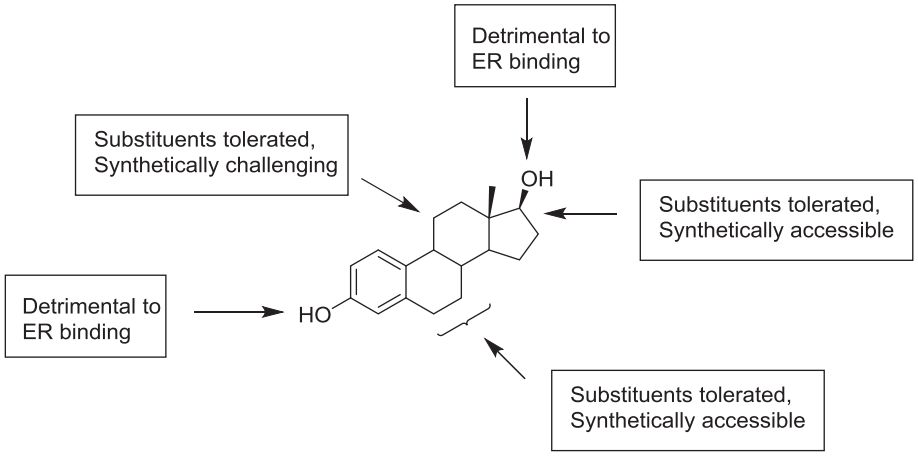


Fig. 1. Basic structure activity relationships for substituted estradiol derivatives.

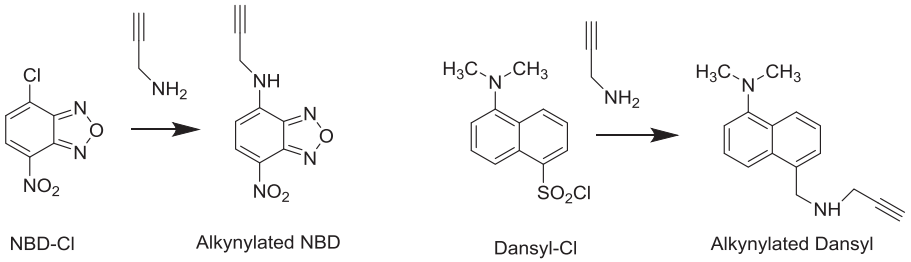


Fig. 2. Functionalization of NBD- and Dansyl-fluorophores.

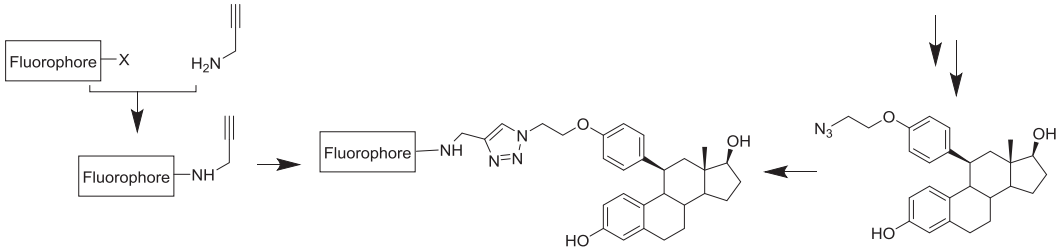


Fig. 3. Convergent synthesis of fluorescently labeled steroidal antiestrogen conjugate.

specific functional groups on intracellular localization (affinity and selectivity) we included the estrone derivative (17-keto) which would have reduced ER affinity, and the estra-4,9-dien-3,17-dione analog which would lack affinity for the ER as a control agent. As fluorescent coupling partners we selected the propargylamine derivatives of NBD- and dansyl fluorophores, components that have been used by others for cellular imaging [15,16]. Coupling of the two components could then be achieved using the [3 + 2} copper-assisted azide- alkyne cyclization (CAAC) reaction [21–23].

2. Experimental

2.1. General

All reactions were carried out in dry glassware unless otherwise noted. Dry glassware was obtained by heating in a laboratory oven at 113 °C overnight (minimum of 12 h). After removal, all glassware was sealed with a rubber septum and placed under a steady stream of dry N_2 via a 20 gauge 1.5 in. needle and cooled to ambient temperature.

All reactions were carried out under an inert (argon) atmosphere with freshly distilled solvents unless otherwise noted·THF was distilled from sodium-benzophenone immediately prior to use. Dichloromethane was distilled from calcium hydride prior to use and methanol was dried by heating at reflux with magnesium turnings and then distilled onto activated, crushed 4 Å molecular sieves. Other anhydrous solvents were purchased as extra dry (< 10 ppm water) and stored over molecular sieves. The starting material, estra-5(10), 9(11)-diene-3,17-dione 3-ethylenedioxy ketal, was purchase from Shanghai Richem International Co and analyzed prior to use. All other reagents were obtained from Sigma-Aldrich Chemical Company or Fisher Scientific and used as provided.

^{1}H NMR and ^{13}C spectra were obtained on a Varian mercury 400 (400 MHz) and are reported in parts per million (ppm). All coupling values (J) are reported in Hz. The NMR spectra were processed using MestReNova version 6.1.0–6224. LC-MS were obtained on Waters e2795 separations module (LC) using a Sunfire C18 column and Waters micromass 7Q (MS). IR spectra were obtained on a Bruker alpha –e ATR spectrometer. Analytical thin layer chromatography (TLC) was performed using silica gel 60A precoated sheets (Sigma-Aldrich) and were visualized using 254 nm/ 366 nm UV lamp, in p-anisaldehyde stain where indicated. All column (flash) chromatography was performed on silica gel unless otherwise indicated. All compounds were synthesized in > 95% in purity, which was determined by LC –MS.

2.2. Synthesis of 3,3-ethylenedioxy-5(10)-α-epoxy-estra-9(11)-ene-17-one 2a and 3,3-etheylenedioxy-5(10)-β-epoxy-estra-9(11)-ene-17-one 2b.

3,3-Ethylenedioxy -estra-5(10),9(11)-diene-17-one **1** (3.0 g, 9.6 mmol) was dissolved in 8 mL of dichloromethane. Hexafluoroacetone (175 μL (1.4 mmol), pyridine (77 μL, 0.96 mmol), and hydrogen peroxide (50%, 1.5 mL, 49.1 mmol) were added at 0 °C. After 18 hrs. aqueous sodium thiosulfate (2 g in 50 mL) was added to quench the reaction. The aqueous layer was extracted with dichloromethane (3 × 100 mL) Organic fractions were combined, dried over magnesium sulfate (anhydrous), filtered and evaporated to dryness The colorless residue was triturated with ether to give a white solid that was collected by filtration and identified by ^{1}H NMR as the α-epoxide **2a**. The filtrate contained a mixture of the two epoxides which was separated using flash chromatography. The desired product **2a** eluted first with hexane–ethyl acetate (7:3). This product was combined with the initial precipitate to give an overall isolated yield of 2.2 g (6.6 mmol, 68%). The β-epoxide was isolated in subsequent fractions to give an isolated yield of 0.4 g (1.2 mmol, 13%).

2a. ^{1}H NMR (400 MHz, CDCl3) δ 6.00 (s, 1H), 3.86 (m, 4H), 2.41 (m, 2H), 2.15 – 1.98 (m, 6H), 1.93 – 1.81 (m, 3H), 1.77 – 1.56 (m, 2H), 1.50 (d, J = 11.0 Hz, 4H), 1.18 (qd, J = 12.4, 3.2 Hz, 1H), 0.82 (s, 3H). ^{13}C NMR (100 MHz, CDCl3) δ 220.10, 136.43, 125.54, 106.76, 64.15, 63.95, 61.44, 59.90, 46.50, 46.15, 40.09, 36.92, 35.77, 33.45, 31.42, 27.88, 24.92, 22.04, 21.75, 14.59 LC -MS – *m/z* - observed M + 1 – 331.42

2.3. Synthesis of 11β-(4-hydroxyphenyl)-estra-4,9-diene-3,17-dione 4

Freshly distilled THF (50 mL) was added to a round bottom flask containing magnesium turnings (2.5 g, 104 mmol, oven-dried overnight). A granule of iodine was added to effect a deep reddish brown color. At this point, of 4-bromophenoxy trimethylsilane **3** (10 mL, 51 mmol) was added dropwise, 1 mL initially, and subsequently 0.5 mL aliquots every 15 min. After addition of 5 mL of **3**, the reaction mixture was gently warmed to 60 °C. The color of the reaction changed from the initial reddish brown to yellow and eventually to a metallic grey. 3,3-Ethylenedioxy-5(10)-α-epoxy-estra-9(11)-ene-17-one **2a** (2.1 g, 6.3 mmol) was dissolved in 15 mL of anhydrous THF. Cu (I) iodide (0.16 g, 0,84 mmol) was added to the solution and the mixture was cooled to −10° C. The Grignard reagent was added dropwise and the resultant mixture was warmed to ambient temperature. After 18 h the reaction was quenched by the addition of aqueous ammonium chloride and ethyl acetate (total 70 mL). The organic layer was separated, washed twice with water (40 mL), dried over magnesium sulfate (anhyd), filtered and evaporated to dryness to give a crude oil. The oil was dissolved in acetic acid- water (20 mL, 7:3 by volume) and the solution was heated at 60° C for 1.5 h. The reaction was diluted with ethyl acetate (20 mL) and neutralized by the addition of aqueous sodium bicarbonate (saturated). The organic phase was separated and the aqueous phase was extracted with ethyl acetate (2 X 20 mL). The organic layers were combined, washed with brine, dried over magnesium sulfate, filtered and evaporated to dryness. The crude product was purified by flash chromatography (7:3 hexane: ethyl acetate) to give 11β-(4-hydroxyphenyl)-estra-4, 9-diene-3,17-dione 4 (1.07 g, 3.0 mmol) in a 47% yield for two steps.

Rf = 0.2 (hexane : ethyl acetate 1:1) ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.84 (s, 1H), 6.93 (d, J = 8.5 Hz, 2H), 6.73 (d, J = 8.5 Hz, 2H), 5.76 (s, 1H), 2.75–2.64 (m, 1H), 2.62–2.54 (m, 3H), 2.48–2.21 (m, 6H), 2.14 – 2.02 (m, 2H), 1.97 – 1.94 (m, 1H), 1.89–1.84 (dd, J = 7.1 Hz, 1H), 1.61–1.43 (m, 3H), 0.51 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 220.81, 200.59, 157.46, 154.66, 146.15, 134.72, 129.65, 127.78, 122.81, 115.59, 50.43, 47.67, 39.53, 37.90, 37.75, 36.50, 35.42, 30.82, 26.54, 25.63, 21.78, 14.31. LC -MS – *m/z* - observed M + 1 – 363.24. Retention time – 1.92 min

2.4. Synthesis of 11β-[4-(2-tosyloxyethoxy)phenyl]-estra-4,9-diene-3,17-dione 5

To 11β-(4-hydroxyphenyl)-estra-4a,9-diene-3,17-dione **4** (510 mg, 1.41 mmol) dissolved in acetonitrile (6 mL) was added cesium carbonate (0.71 g, 2.1 mmol). The reaction mixture was stirred for 30 min followed by the addition of ethylene glycol ditosylate (0.35 g, 0.94 mmol). The reaction vessel was sealed and heated at 120° C for 20 h. The reaction mixture was cooled to ambient temperature and poured into a mixture of ethyl acetate and water (100 mL, 1:1 by volume) The organic layer was separated, washed with water (2 × 50 mL), dried over magnesium sulfate (anhyd), filtered and evaporated to dryness. The crude product was purified by flash chromatography (ethyl acetate: hexane, 1:1) to afford afforded **5** (0.40 g, 0.7 mmol) in 53% isolated yield.

Rf = 0.13 (hexane : ethyl acetate 1:1) ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (d, J = 8.0 Hz, 2H), 7.33 (d, J = 7.9 Hz, 2H), 7.05 (d, J = 8.4 Hz, 2H), 6.70 (d, J = 8.5 Hz, 2H), 5.77 (s, 1H), 4.34–4.30 (m, 2H), 4.12 – 4.08 (m, 2H), 2.77–2.67(m, 1H), 2.66 – 2.55 (m, 3H), 2.43 (s, 3H), 2.50 – 2.19 (m, 6H), 2.18 – 1.96 (m, 3H), 1.93 – 1.85 (dd, 1H), 1.66 – 1.46 (m, 3H), 0.52 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ219.96, 218.96, 199.34, 156.25, 156.02, 145.07, 144.83, 136.72, 132.80, 130.14, 129.94, 128.02, 123.46, 114.72, 68.20, 65.47, 50.62, 47.69, 39.58, 37.90, 36.85, 35.47, 30.93, 29.75, 26.80, 25.92, 21.83, 21.14, 14.36. LC -MS – *m/z* - observed M + 1 – 561.42 Retention time – 3.04 min

2.5. Synthesis of 11β-[4-(2-azidoethoxy)phenyl]-estra-4,9-diene-3,17-dione 6

11β-[4-(2-tosyloxyethoxy)phenyl]-estra-4,9-diene-3,17-dione **5** (120 mg, 0.2 mmol) was dissolved in 8 mL of hot ethanol. Sodium azide (55 mg, 0.84 mmol) was added and the reaction was heated at reflux for 18 hrs. The reaction mixture was cooled to ambient temperature poured into 50 mL ethyl acetate. The organic phase was washed with water (3 X 50 mL), dried over magnesium sulfate, filtered and evaporated to dryness The crude product was purified by flash chromatography (hexane: ethyl acetate 1:1) to afford 83 mg (0.19 mmol) of **6**, corresponding to isolated yield of 93%.

Rf = 0.29 (hexane : ethyl acetate 1:1) ^{1}H NMR (400 MHz, CDCl3) δ 7.10 (d, J = 8.4 Hz, 2H), 6.84 (d, J = 8.6 Hz, 2H), 5.79 (s, 1H), 4.11 (t, J = 4.8 Hz, 2H), 3.59 (t, J = 4.7 Hz, 2H), 2.80 – 2.70 (m, 1H), 2.63 (m, 3H), 2.55 – 2.24 (m, 6H), 2.20 – 2.07 (m, 2H), 2.05 – 1.98 (m, 1H), 1.92 (dd, 1H), 1.66 – 1.50 (m, 3H), 0.56 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 230.13, 199.56, 156.60, 156.20, 145.02, 136.80, 130.32, 128.18, 123.64, 114.91, 67.13, 50.81, 50.40, 47.88, 39.76, 38.07, 37.98, 37.02, 35.64, 31.10, 26.98, 26.11, 22.08, 14.65. LC -MS – *m/z* - observed M + 1 – 432.26. Retention time – 2.77 min IR 2109.39 cm^{-1} (azide)

2.6. Synthesis of 3-hydroxy-11β-[4-(2-azidoethoxy)phenyl]-estra-1,3,5(10)-triene-17-one acetate 7

To 11β-[4-(2-azidoethoxy)phenyl]-estra-4,9-diene-3,17-dione **6** (83 mg, 0.19 mmol) dissolved in dichloromethane (5 mL) were added acetic anhydride (18 μL, 0.2 mmol) and acetyl bromide (35 μL, 0.47 mmol). The reaction solution was stirred at ambient temperature for 18 h and then quenched by the addition of aqueous sodium bicarbonate (20 mL). The organic layer was washed with water, dried over magnesium sulfate (anhyd), filtered and evaporated to dryness. (3x 50 mL each). All the aqueous fractions were back extracted with ethyl acetate. The organic fractions were collected and were dried with $MgSO_4$. The crude product was purified using flash chromatography (7:3 hexane: ethyl acetate) to give 67 mg (0.14 mmol) of 7, corresponding to 74% yield.

Rf = 0.18 (7:3 Hexane: Ethyl Acetate) ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.00 – 6.91 (m, 3H), 6.86 (s, 1H), 6.65 (d, J = 8.3 Hz, 2H), 5.29 (s, 1H), 4.07 – 3.96 (m, 2H), 3.61 – 3.46 (m, 2H), 3.07–3.01 (m, 1H), 3.01 –

2.88 (m, 2H), 2.56 – 2.39 (m, 2H), 2.38 – 2.35 (m, 1H), 2.24 (s, 3H), 2.20 – 2.00 (m, 3H), 1.96 (dd, 1H), 1.72 – 1.43 (m, 3H), 0.45 (s, 3H). ^{13}C NMR (100 MHz, CDCl3) δ 220.20, 169.74, 155.51, 148.19, 137.53, 135.66, 130.58, 127.57,121.73, 119.24, 113.89, 66.74, 52.22, 50.28, 48.19, 47.69, 40.03, 38.12, 35.39, 34.94, 30.04, 27.18, 21.33, 15.25. LC -MS – *m/z* - observed M + 1 – 474.30 ; M + 18 – 491.32. Retention time – 3.44 min.

2.7. Synthesis of N-Dansyl propargylamine 8.

To dansyl chloride (55 mg, 0.21 mmol) dissolved in dichloromethane (4 mL) was added sequentially triethyl amine (20 μL, 0.21 mmol) and propargyl amine (40 μL, 0.63 mmol). The reaction was stirred at ambient temperature for 16 h and then evaporated to dryness. The resultant crude material was purified by flash chromatography (hexane:ethyl acetate 3:2) to yield 40 mg (0.14 mmol, 65%) of the final product **8**.

Rf = 0.5 (1:1 hexane: ethyl acetate) ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.56 (d, J = 8.5 Hz, 1H), 8.26 (t, J = 8.3 Hz, 2H), 7.55 (dd, J = 14.8, 7.2 Hz, 2H), 7.19 (d, J = 7.5 Hz, 1H), 4.87 (t, J = 5.9 Hz, 1H), 3.77 (dd, J = 6.0, 2.5 Hz, 2H), 2.89 (s, 6H), 1.91 (s, 1H) ^{13}C NMR (100 MHz, $CDCl_3$) δ 152.19, 134.24, 130.97, 130.10, 129.98, 129.89, 128.73, 123.31, 118.61, 115.34, 77.86 72.84, 45.55, 33.16 LC -MS – *m/z* - observed M + 1 – 289.17. Retention time 2.13 min

2.8. Synthesis of 7-nitro-N-(prop-2-yn-1-yl)benzo[c][1,2,5] oxadiazol-4-amine (9)

To 4-chloro-7-nitrobenzo[c][1,2,5] oxadiazole, (75 mg 0.37 mmol) dissolved in 3 mL THF, were added propargyl amine (26 μL, 0.40 mmol), cesium carbonate (136 mg, 0.42 mmol). The reaction mixture was stirred at ambient temperature for 18 h. The reaction mixture was poured into ethyl acetate (50 mL) and washed with water (2 × 25 mL), dried over magnesium sulfate (anhydrous), filtered and evaporated to dryness. The crude material was purified by flash chromatography (hexane : ethyl acetate 3:2) to give 53 mg (0.24 mmol, 65% yield) of **9**.

Rf = 0.17 (hexane : ethyl acetate 7:3) ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.54 (d, J = 8.5 Hz, 1H), 6.37 (t, J = 12.5 Hz, 2H), 4.31 (d, J = 3.7 Hz, 3H), 2.43 (s, 1H), 1.62 (s, 1H)

2.9. Synthesis of 11β-(4-(2-(4-(5-dimethylamino) naphthalene)-1-sulfonamido)-methyl)-1H-1,2,3- triazol-1-yl)-ethoxy)phenyl) estra 4,9-diene-3,17-dione (10a)

11β-[4-(2-azidoethyoxy)phenyl]-estra-4,9-diene-3,17-dione, **6**, **(**13 mg, 0.03 mmol) was dissolved in *tert*-butanol : water (1:1, 4 mL). *N*-Dansyl propargylamine, **8,** (10 mg, 0.03 mmol), sodium ascorbate (350 μL, 0.035 mmol in a 0.1 mmol/mL solution) and copper (II) sulfate, (70 μL, 0.007 mmol in a 0.1 M solution) were added. The reaction vessel was sealed and heated at 80 °C for 16 hrs. The reaction was cooled and partitioned between ethyl acetate and water. The organic phase was washed sequentially with water and brine, dried over magnesium sulfate (anhydrous), filtered and evaporated to dryness. The crude material was purified by flash chromatography (ethyl acetate : hexane 9:1) to obtain 14 mg (0.019 mmol, 66% yield) of 11β-(4-(2-(4-(aminomethyl)-1H-1,2,3- triazol-1-yl)-ethoxy)phenyl) estra-4,9-diene-3,17-dione **10a**.

Rf = 0.13 (9:1 ethyl acetate: hexane) ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.45 (d, J = 8.5 Hz, 1H), 7.85 (s, 1H), 7.08 (d, J = 8.3 Hz, 2H), 6.97 (s, 1H), 6.74 (d, J = 8.4 Hz, 2H), 6.34 (d, J = 8.5 Hz, 1H), 5.79 (s, 1H), 4.83–4.78 (d, 2H), 4.40 – 4.27 (m, 2H), 3.34 (s, 1H), 2.77–2.67 (m, 1H), 2.66 – 2.57 (m, 3H), 2.53 – 2.32 (m, 6H), 2.31–2.20 (m, 2H), 2.19 – 2.12 (m, 2H), 1.96 – 1.88 (dd, 1H), 0.95 – 0.80 (m, 3H), 0.51 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 218.88, 199.22, 155.85, 151.96, 144.56, 143.72, 137.06, 134.30, 130.12, 130.12, 129.80, 129.59, 129.49, 128.61, 128.03, 123.37, 123.07, 118.48, 115.28, 114.62, 66.04, 50.52, 49.64, 47.59, 45.39, 39.48, 38.79, 37.88, 37.82, 36.74, 35.36, 30.82, 26.70, 25.84, 21.80, 14.43. LC -MS – *m/z* - observed M + 1 – 720.27. Retention time 2.66 min IR 1735.46 cm^{-1} (17C = O), 1658.35 cm^{-1} (3C = O)

2.10. Synthesis of 11β-(4-(2–4(-(((7-nitrobenzo[c][1,2,5] oxadiazol-4-yl) amino)methyl-1H-1,2,3-triazol-1-yl)ethoxy)phenyl)-estra-4,9-diene-3,17-dione (10b)

11β-[4-(2-azidoethyoxy) phenyl]-estra-4,9-diene-3,17-dione, **6**, (16 mg 0.04 mmol) of was dissolved in a solution of *tert* butanol-water (1:1, 5 mL v/v). 7-Nitro-N-(prop-2-yn-1-yl)benzo[c][1,2,5] oxadiazol-4-amine **9** (15 mg, 0.06 mmol) sodium ascorbate (350 μL 0.035 mmol in a solution of 0.1 mmol/mL), copper (II) sulfate, (70 μL, 0.007 mmol in a solution of 0.1 mmol/mL) were added and the reaction vial was sealed. The reaction was heated at 80 °C with stirring for 18 h, cooled to ambient temperature and partitioned between ethyl acetate and water. The organic phase was washed with water (2 × 20 mL), brine, dried over magnesium sulfate (anhydrous), filtered and evaporated to dryness. The crude solid was purified by flash chromatography (ethyl acetate : hexane, 9:1) to give the pure product **10b** (12 mg, 0.018 mmol) in 46% isolated yield.

Rf = 0.04 (9:1 ethyl acetate: hexane) ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.45 (d, J = 8.5 Hz, 1H), 7.85 (s, 1H), 7.26 (s, 1H), 7.08 (d, J = 8.3 Hz, 2H), 6.97 (s, 1H), 6.74 (d, J = 8.4 Hz, 2H), 6.35 (d, J = 8.5 Hz, 1H), 4.81 (d, J = 18.5 Hz, 2H), 4.44 – 4.25 (m, 2H), 3.34 (s, 1H), 2.77 – 2.68 (m, 1H), 2.54 – 2.0 (m, 9H), 1.96 – 1.88 (dd, 1H), 1.92 (dd, J = 13.7, 7.2 Hz, 1H), 1.61 – 1.50 (m, 3H), 0.96 – 0.78 (m, 3H), 0.51 (s, 3H). ^{13}C NMR (100 MHz, acetone) δ 217.99, 198.12, 157.14, 156.54, 145.82, 138.39, 137.65, 130.66, 129.08, 124.69, 123.58, 115.42, 67.28, 60.53, 51.36, 50.42, 48.16, 40.36, 39.01, 38.76, 37.51, 35.68, 31.33, 27.73, 26.65, 22.41, 20.83, 14.85, 14.50. LC -MS – *m/z* - observed M + 1 – 650.21. Retention time 2.34 min. IR = 1733.58 cm^{-1} (17C = O), 1657.42 cm^{-1} (3C = O)

2.11. Synthesis of 3-hydroxy-11β-(4-(2-(4((5-dimethylamino) naphthalene)-1-sulfonamido)-methyl)-1H-1,2,3- triazol-1-yl)-ethoxy) phenyl) estra-1,3,5(10)-triene-17-one-diene (11a)

3-Hydroxy-11β-[-4-(2-azidoethoxy)-phenyl]-estra-1,3,5(10)-triene-17-one acetate **7** (36 mg, 0.08 mmol) was dissolved in *tert*-butanol – water (6 mL, 1:1 solution). *N*-Dansyl propargylamine, **8a**, (33 mg, 0.1 mmol), sodium ascorbate (350 μL, 0.035 mmol in a solution of 0.1 M), copper (II) sulfate (70 μL, 0.007 mmol in a solution of 0.1 M) were added to the reaction vial, sealed and the reaction was heated at 90 °C for 18 h. The reaction was cooled and partitioned between ethyl acetate and water. The organic phase was washed sequentially with water and brine, dried over magnesium sulfate (anhyd), filtered and evaporated to dryness. The crude material was purified by flash chromatography (step gradient from 1:1 to 4:1 ethyl acetate: hexane) to give the pure acetylated intermediate (40 mg, 0.052 mmol, 75%).

The acetylated intermediate (20 mg, 0.03 mmol) was dissolved in methanol (4 mL) and saponified by the addition of 4.2 mg (0.07 mmol) of potassium hydroxide. Isolation of the product yielded **10a** (18 mg, 99%)

Rf = 0.16 (7:3 hexane: ethyl acetate) ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.42 (d, J = 8.5 Hz, 1H), 8.17 (t, J = 7.8 Hz, 2H), 7.44 (t, J = 8.0 Hz, 1H), 7.37 (t, J = 7.9 Hz, 1H), 7.32 (s, 1H), 7.11 (d, J = 7.5 Hz, 1H), 6.98 (d, J = 8.1 Hz, 2H), 6.75 (d, J = 8.4 Hz, 1H), 6.62 (s, 1H), 6.54 (d, J = 8.2 Hz, 2H), 6.38 (d, J = 7.9 Hz, 1H), 5.74 (s, 1H), 5.29 (s, 1H), 5.11 (s, 1H), 4.43 (s, 2H), 4.15 (d, J = 5.5 Hz, 2H), 4.05 (d, J = 4.3 Hz, 2H), 3.97 (s, 1H), 2.85 (s, 6H), 2.47 (d, J = 14.9 Hz, 2H), 2.30 (d, J = 11.2 Hz, 2H), 2.18 – 2.03 (m, 3H), 2.00 – 1.90 (m, 1H), 1.67 – 1.40 (m, 3H), 0.43 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) 154.84, 153.54, 151.80, 137.65, 136.76, 134.60, 130.84, 130.62, 129.75, 129.65,

129.55, 128.69, 127.63, 123.27, 118.82, 115.59, 115.41, 113.79, 113.65, 65.81, 52.15, 49.78, 48.34, 47.41, 45.55, 39.98, 38.53, 38.24, 35.48, 35.18, 30.11, 29.83, 27.34, 21.48, 15.37, −29.25. LC -MS – *m/z* - observed M + 1 – 720.28. Retention time 2.83 min. IR = 1735.46 cm^{-1} (17C = O)

2.12. Synthesis of 3-hydroxy-11β-(4-(2–4(-(((7-nitrobenzo[c][1,2,5] oxadiazol-4-yl)amino)methyl-1H-1,2,3-triazol-1-yl)ethoxy)phenyl)-estra-1,3,5(10)-triene-17-one 11b

3-Hydroxy-11β-[-4-(2-azidoethoxy)-phenyl]-estra-1,3,5(10)-triene-17-one acetate **(7)** (20 mg, 0.04 mmol) was dissolved *tert*- butanol – water (5 mL, 1:1 v/v). 7-nitro-N-(prop-2-yn-1-yl)benzo[c][1,2,5] oxadiazol-4-amine, **9a**, (18 mg, 0.08 mmol), sodium ascorbate, (350 μL, 0.035 mmol – 0.1 M solution), copper (II) sulfate (70 μL, 0.007 mmol – in a 0.1 M solution) were added and the reaction vessel was sealed. The reaction was heated to 80 °C and stirred for 18 h. The reaction was cooled to ambient temperature and partitioned between ethyl acetate and water. The organic phase was washed with water (2 × 20 mL), brine, dried over magnesium sulfate (anhydrous), filtered and evaporated to dryness. The crude solid was purified by flash chromatography (ethyl acetate: hexane, 4:1) to give 21 mg of the pure intermediate. 10 mg (0.01 mmol) of the intermediate was dissolved in methanol (4 mL) and saponified by the addition of potassium hydroxide (4.2 mg, 0.07 mmol). After stirring for 2 h, the reaction was quenched by the addition of glacial acetic acid and partitioned between ethyl acetate and water. The organic phase was washed with water (2 × 20 mL), brine, dried over magnesium sulfate (anhydrous), filtered and evaporated to dryness. The crude solid was purified by flash chromatography (ethyl acetate : hexane, 4:1) to give 18 mg of the pure product in 90% isolated yield.

Rf = 0.28, 100% Ethyl acetate ^{1}H NMR (400 MHz, CDCl3) δ 8.37 (d, J = 8.5 Hz, 1H), 7.81 (s, 1H), 7.09 – 7.01(m, 1H), 6.97 (s, 1H), 6.73 (d, J = 8.5 Hz, 2H), 6.60 (s, 1H), 6.53 (d, J = 8.2 Hz, 1H), 6.37 (d, J = 8.2 Hz, 1H), 6.33 (d, J = 8.6 Hz, 1H), 4.79 (d, J = 5.1 Hz, 2H), 4.71 (s, 2H), 4.21 (s, 2H), 3.37 (s, 1H), 2.93 – 2.81 (m, 1H), 2.55 – 1.9 (m, 9H), 1.70 – 1.42 (m, 3H), 0.92–0.78 (m, 2H), 0.40 (s, 3H) ^{13}C NMR (100 MHz, d_6-acetone) δ 218.11, 156.36, 155.75, 138.42, 137.75, 131.76, 129.97, 128.40, 124.75, 116.20, 114.55, 114.23, 77.41, 67.20, 60.68, 56.09, 52.95, 50.58, 48.84, 48.39, 41.21, 39.18, 36.16, 35.69, 30.89, 28.28, 22.06, 15.79, 14.65. LC -MS – *m/z* - observed M + 1 – 650.18. Retention time 2.57 min. IR = 1733.29 cm^{-1} (17C = O)

2.13. Synthesis of 11β-(4-(2-(4-(((5-dimethylamino) naphthalene)-1-sulfonamido)-methyl)-1H-1,2,3-triazol-1-yl)ethoxy)phenyl)-estra-1,3,5(10)-triene-3,17β-diol (12a)

To 3-hydroxy-11β-(4-(2-(4-(((5-dimethylamino) naphthalene)-1-sulfonamido)-methyl)-1H-1,2,3-triazol-1-yl)ethoxy)phenyl)-estra-1,3,5(10)-triene-17-one-3-acetate **10** (20 mg 0.03 mmol) dissolved in methanol (4 mL) was added sodium borohydride (1.8 mg, 0.05 mmol) at 0°c. The reaction stirred for 4 h, warming to ambient temperature. Potassium hydroxide in methanol (4.2 mg, 2 mL) was added and the reaction was stirred for an additional 16 h. The reaction was quenched by the addition of glacial acetic acid and partitioned between ethyl acetate and water. The organic fraction was washed with water (2 × 10 mL), dried over magnesium sulfate (anhydrous), filtered and evaporated to dryness. The product was isolated in essentially quantitative yield (14 mg, 0.019 mmol, 99%)

Rf = 0.18 (7:3 ethyl acetate: hexane) ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.42 (d, J = 8.5 Hz, 1H), 8.17 (t, J = 8.0 Hz, 2H), 7.40 (dt, 7.9 Hz, 2H), 7.28 (s, 1H),7.09 (d, J = 7.5 Hz, 1H), 6.95 (d, J = 8.0 Hz, 2H), 6.72 (d, J = 8.5 Hz, 1H), 6.59 (s, 1H), 6.50 (d, J = 8.1 Hz, 2H), 6.36 (d, J = 7.9 Hz, 1H), 5.76 (s, 1H), 5.11 (s, 1H), 4.39 (s, 2H), 4.12 (dd, 6.8 Hz, 4H), 4.01 (s, 2H), 3.88 (s, 1H), 3.66 (m, 1H), 3.34 (s, 1H), 2.84 (s, 6H), 2.46 (d, J = 12.7 Hz, 1H), 2.18 – 1.95 (m, 5H), 1.80 – 1.63 (m, 1H), 0.94 – 0.77 (m, 3H), 0.30 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 154.64, 153.37, 151.88, 137.87, 137.26, 134.56, 130.92, 130.65, 130.34, 129.78, 129.69, 129.57, 128.70, 127.64, 123.28, 118.78, 115.52, 115.39, 113.61, 113.48, 65.83, 60.57, 51.79, 49.76, 47.32, 45.54, 43.76, 38.65, 38.45, 35.55, 30.26, 29.84, 28.03, 23.28, 21.21, 14.33. LC -MS – *m/z* - observed M + 1 – 722.27. Retention time – 2.61 min. IR = 3296.03 cm^{-1}, 3265.35 cm^{-1}

2.14. Synthesis of 11 β – (4-(2-(4-(((7-nitrobenzo[c][1,2,5] oxadiazol-4-yl)amino)methyl)-1H-1,2,3-triazol-1-yl-ethoxy)phenyl)-estra-1,3,5(10) trien-3,17β-diol (12b)

To 11 β – (4-(2-(4-(((7-nitrobenzo[c][1,2,5] oxadiazol-4-yl)amino) methyl)-1H-1,2,3-triazol-1-yl-ethoxy)phenyl)-estra-1,3,5(10)trien-17-one acetate **11e** (10 mg, 0.03 mmol) dissolved in methanol (4 mL) at 0 °C, was added sodium borohydride (1 mg, 0.03 mmol) and the reaction was warmed to ambient temperature and stirred for an additional 2 h. Potassium hydroxide (5 mg, 0.07 mmol) was added and the reaction was stirred for an additional16h. The reaction was quenched by the addition of glacial acetic acid and partitioned between ethyl acetate and water. The organic phase was washed with water (2 × 20 mL), brine, dried over magnesium sulfate (anhydrous), filtered and evaporated to dryness. The crude solid was purified by flash chromatography (ethyl acetate: hexane 9:1) to give the pure product (7 mg, 0.01 mmol) in 74% yield.

Rf = 0.22, 100% ethyl acetate ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.43 (t, J = 8.6 Hz, 1H), 7.77 (s, 1H), 6.95 (d, J = 8.1 Hz, 1H), 6.86 (s, 1H), 6.76 (d, J = 8.5 Hz, 2H), 6.58 (s, 1H), 6.53 (d, J = 8.3 Hz, 1H), 6.35 (dd, J = 14.5, 8.5 Hz, 2H), 4.79 (s, 2H), 4.72 (s, 2H), 4.23 (s, 2H), 3.35 (s, 1H) 3.04–2.92 (m, 1H), 2.90 – 2.74 (m, 2H), 2.57 (s, 1H), 2.45 (dd, 1H), 2.37 – 2.29 (m, 1H), 2.21 (dd, 1H), 2.11, (d, 1H), 2.00 (m, 1H), 1.82 – 1.48 (m, 7H), 0.88 (m, 2H), 0.28 (s, 3H). ^{13}C NMR (100 MHz, d_6-acetone) δ 156.19, 155.64, 137.87, 131.83, 128.44, 124.72, 116.17, 114.38, 111.08, 82.70, 77.44, 67.23, 56.12, 52.87, 50.63, 48.46, 46.79, 39.52, 36.66, 31.08, 24.01, 13.81, 1.58. LC -MS – *m/z* - observed M + 1 – 652.15. Retention time 2.41 min IR = 3321.91 cm^{-1}

2.15. Determination of fluorescence spectra

2.15.1. Absorbance spectra

Absorbance spectra of all the compounds were recorded on a Thermo Scientific Genesys 10S UV–VIS spectrometer, scanning from 200 nm to 800 nm. All the compounds were dissolved in methanol. Each compound was background corrected.

For each compound, 5 spectra were taken at decreasing concentrations (generally 10 μg/mL-2 μg/mL). Spectra were normalized (divided by the maximum absorption) and the average absorbance for each wavelength was taken for the final spectrum.

2.15.2. Emission spectra

Emission spectra were recorded on a Hitachi F-2000 Fluorescence Spectrometer. Compounds containing NBD fluorophore were excited at 465 nm and compounds containing dansyl fluorophore were excited at 340 nm. The emissions spectra were recorded from 300 nm to 800 nm. For each compound, 5 spectra were taken at decreasing concentrations (generally 1.0 μg/mL-0.2 μg/mL). Spectra were normalized (divided by the maximum emission) and the average absorbance for each wavelength was taken to generate the final spectrum

3. Results and discussion

3.1. Chemistry

The design of the fluorescent probes for the estrogen receptor was based on integrating a high-affinity steroidal ligand with small, neutral fluorophores. [Fig. 3] The use of the steroidal as opposed to

nonsteroidal ligands was based on reports that steroidal ligands not only have higher ER affinity, but also possess greater selectivity for the steroid hormone receptors. As such they would reduce binding to off target proteins. We have demonstrated previously that the 11β-(4-oxyphenyl)estradiol scaffold retains a high affinity (RBA > 25%) for the estrogen receptor, and that attaching (substituted) alkyl groups to the oxygen do not diminish binding affinity, but beyond the methyl group, confer antagonist properties.[ ] In addition, the method for preparing the final compounds allows for synthesis of structurally related compounds to test the influence of functional groups on intracellular localization. The presence of the 17-keto group reduces the ER binding affinity by an order of magnitude, and the de-aromatization of the A-ring essentially eliminated ER-binding. The use of the 2-azidoethyl linker would provide three properties to the final products. First, the azidoethyl group confers antagonist properties to the steroidal ligand which would generate a novel probe for interrogating ER-containing cells. Second, the azide provides a facile click method for conjugating the steroidal component to a complementary alkynylated partner, in this case a fluorescent component. Third, the clicked product would have the fluorescent group extended into the solvent space in the ligand-receptor complex as the triazole would occupy the same site as the tertiary amine of the aminoethoxy nonsteroidal antiestrogens. Because this initial study was to demonstrate proof of concept, physicochemical properties were more important than imaging characteristics for the fluorescent component. Both the dansyl and NBD fluorophores are relatively low molecular weight and neutral groups. As such they are more compatible with the chemistries of the steroid component than inherently charged fluorescein, rhodamine or cyanine agents. Also, conversion to propargylated derivatives was simple and the products were easy to purify and store. Conjugation with the complementary azido reagents using the copper (I)-assisted cycloaddition method is robust and would give the final products in good yields. If the initial set of fluorescent probes were to successfully demonstrate selective ER-dependent localization, then subsequent studies could extend the methods to include variation on the linker attached to the (4-oxyphenyl) group, different fluorophores as well as different conjugation chemistries.

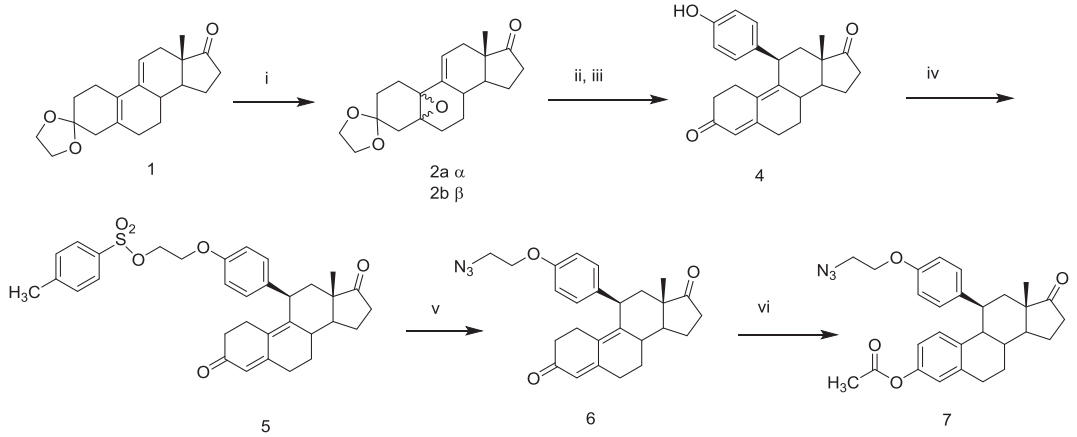
The synthesis of the steroidal scaffold was accomplished using our previously described method (Scheme 1). Starting with commercially available diene-dione monoketal **1**, epoxidation with hydrogen peroxide under basic conditions gave the 5,10-α-epoxide **2a** as the major product (5.5:1.0 α:β isomers) which was isolated by flash chromatography in a 68% yield. Cu(I)-mediated 1,4-Grignard addition of the silylated phenolic reagent **3**, followed by acidic hydrolysis, deprotection and elimination gave the 11β-(4-hydroxyphenyl)-estra-4,9-diene-3,17-dione **4** in a 47% isolated yield for the combined two-steps. Alkylation with ethylene glycol ditosylate provided the monotosylate intermediate **5** in a 53% isolated yield and which was converted to the corresponding azidoethoxyphenyl derivative **6** in a 93% yield. This intermediate would constitute the scaffold for the control steroidal derivatives which would lack affinity for the estrogen receptor. Aromatization with acetic anhydride-acetyl bromide in dichloromethane smoothly converted the diene-dione derivative to the aromatic estrone **7** in a 74% isolated yield.

As previously noted, the criteria for the fluorophoric components primarily focused on their being low molecular weight, stable, neutral molecules. Preparation of the fluorescent coupling partners was achieved using relatively simple coupling reactions. (Scheme 2) Reaction of propargyl amine with dansyl chloride under basic conditions gave a 65% isolated yield of dansylated amine **8**. Displacement of the chloro-substituent of NBD-Cl by propargyl amine in methanol provided the corresponding NBD-propargyl amine **9** in a 65% isolated yield.

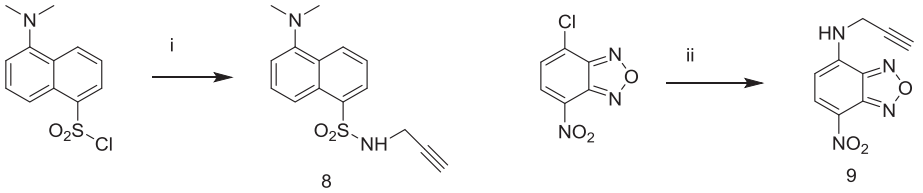
The two components were then coupled using the Cu(I)-assisted azide-alkyne coupling (CAAC) chemistry to generate the set of fluorescently labeled steroids. (Scheme 3) Synthesis of the control compounds **10a** and **10b** was accomplished in one step using the CAAC reaction with the azido-steroidal diene-dione **6** and the propargylated fluorophores **8** and **9**. Flash chromatography of the crude product gave the pure fluorescent derivatives **10a** and **10b** in 66% and 46% yields, respectively. Coupling of the acetylated steroidal ketone **7** with the propargylated fluorophores, followed by saponification of the 3-acetate gave, after chromatographic purification, the final products **11a** and **11b** in 75% and 90% yields. Repetition of the first step, followed by reduction of the ketone and saponification of the ester, gave after chromatographic purification, the fluorescent high affinity steroidal antagonists **12a** and **12b** in 99% and 74% yields. All products were characterized by LC-MS, ^{1}H-and ^{13}C NMR for purity and identity.

3.2. Fluorescence studies.

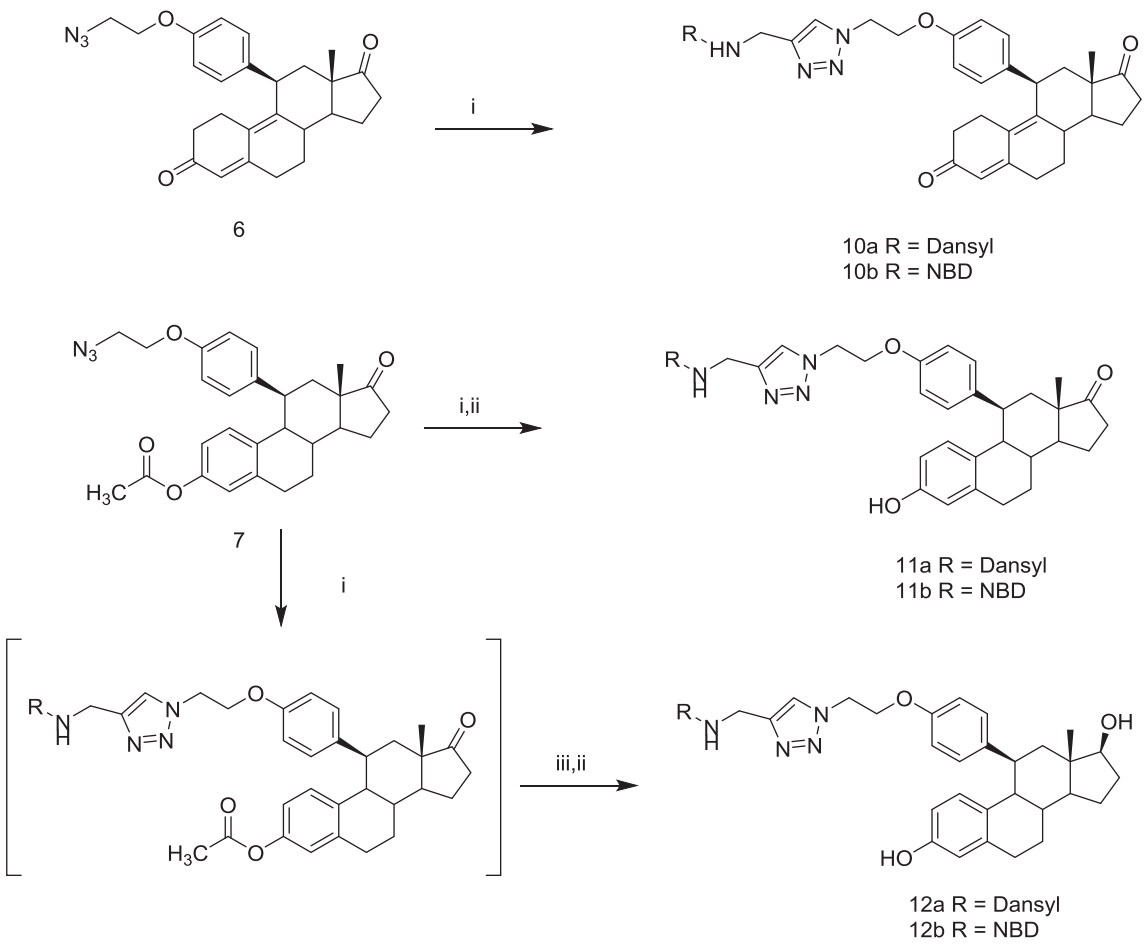
The set of six fluorescent derivatives was evaluated for its fluorescent characteristics [Table 1]. In all cases, the compounds retained the excitation and emission properties associated with their respective dansyl and NBD-fluorophores [24,25]. The excitation maximum for the dansylated steroids was at approximately 340 nm and its major emission peak was 515 nm. For the NBD-steroids, excitation occurred at approximately 470 nm with peak emission at 520 nm. This is similar to the reported propargylamine derivatives of dansyl and NBD [24,25] and other comparably labeled compounds [15,16]. The maxima are somewhat sensitive to the solvent used but the overall spectra were quite similar. The presence of the steroidal component, whether aromatic **11a/b**, **12a/b** or conjugated dien-one system **10a/b**, did not



Scheme 1. Synthesis of functionalized steroid derivatives 6 and 7. Reagents and conditions: (i) CF_3COCF_3, 50% H_2O_2, pyridine, CH_2Cl_2, 0 °C, 18 h, 68% yield α-isomer; (ii) a. 4-bromophenoxy trimethylsilane **3**, Mg; b. Cu(I)I, THF, −10 °C to RT; c. aq. NH_4Cl ; (iii) CH_3CO_2H-H_2O (7:3), 60 °C, 1.5 h; (iv) $TsOCH_2CH_2OTs$, Cs_2CO_3, CH_3CN, 120 °C, 20 h, sealed tube; (v) NaN_3, ethanol, reflux, 18 h; (vi) acetic anhydride, CH_3COBr, CH_2Cl_2, R.T., 18 h.



Scheme 2. Synthesis of fluorescent conjugating groups 8 and 9. Reagents and conditions: (i) propargyl amine, TEA, CH_2Cl_2, R.T., 18 h; (ii) propargyl amine, Cs_2CO_3, THF, R.T., 18 h.



Scheme 3. Synthesis of fluorescently labeled steroidal antiestrogen and steroidal control. Reagents and conditions: (i) $CuSO_4$, sodium ascorbate, **8**/**9**, *tert*-BuOH-H2O, 90 °C, 18 h; (ii) a. KOH, CH_3OH; b. CH_3CO_2H; (iii) $NaBH_4$, CH_3OH.

Table 1
Excitation and Emission maxima for fluorescently labeled steroids.

Compound	Excitation maximum (nm)	Emission maximum (nm)
10a Diene-dione-dansyl	340	515
10b Diene-dione-NBD	470	520
11a Estrone-dansyl	340	515
11b Estrone-NBD	470	520
12a Estradiol-dansyl	360	515
12b Estradiol-NBD	470	520

Spectra determined in methanol.

produce any quenching or alteration of the fluorophore. This suggests that the location of the fluorescent moiety is insulated from the pharmacophoric steroidal scaffold by the 11β-oxyphenyl moiety.

In summary, we have described the design and successful synthesis of a new class of fluorescently labeled estrogen receptor-targeted compounds. Starting from the steroidal estrogenic core, we have introduced a substituent at the 11β-position that can be readily modified to accept a large fluorophore without compromising receptor binding affinity. The fact that the substituent also changes the pharmacology from estrogen to antiestrogen provides an intriguing aspect as it is largely unknown what effect that has upon receptor localization. The synthetic chemistry used to introduce the substituent also permits generating analogs or derivatives with lower affinity for the receptor, thereby providing for internal controls. Although the initial fluorophores-NBD and dansyl-are not considered optimal for receptor imaging, the fact that they are small and neutral groups allows them to serve as potential proof-of-concept agents. Analysis of the absorption and emission spectra indicated that the fluorophore is insulated from the pharmacophore, and should permit successful in vitro imaging of the compounds. Because the site of ligation is at the receptor-solvent interface, if these agents can successfully image estrogen receptor density within the cells, other fluorophores with better imaging properties, but which are either larger or charged, can be evaluated in future studies.

The evaluation of the labeled steroids as ER ligands and the use of this series of fluorescently labeled steroids to evaluate ER-dependent localization in breast cancer cell lines are currently in progress. The results of those studies will be presented in a future publication.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.steroids.2019.02.013.

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